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Sequence Analysis of Herpes Simplex Virus 1 Thymidine Kinase and DNA Polymerase Genes from over 300 Clinical Isolates from 1973 to 2014 Finds Novel Mutations That May Be Relevant for Development of Antiviral Resistance

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A total of 302 clinical herpes simplex virus 1 (HSV-1) strains, collected over 4 decades from 1973 to 2014, were characterized retrospectively for drug resistance. All HSV-1 isolates were analyzed genotypically for nonsynonymous mutations in the thymidine kinase (TK) and DNA polymerase (Pol) genes. The resistance phenotype against acyclovir (ACV) and/or foscarnet (FOS) was examined in the case of novel, unclear, or resistance-related mutations. Twenty-six novel natural polymorphisms could be detected in the TK gene and 69 in the DNA Pol gene. Furthermore, three novel resistance-associated mutations (two in the TK gene and one in the DNA Pol gene) were analyzed, and eight known but hitherto unclear amino acid substitutions (two encoded in TK and six in the DNA Pol gene) could be clarified. Between 1973 and 2014, the distribution of amino acid changes related to the natural gene polymorphisms of TK and DNA Pol remained largely stable. Resistance to ACV was confirmed phenotypically for 16 isolates, and resistance to ACV plus FOS was confirmed for 1 isolate. Acyclovir-resistant strains were observed from the year 1995 onwards, predominantly in immunosuppressed patients, especially those with stem cell transplantation, and the number of ACV-resistant strains increased during the last 2 decades. The data confirm the strong genetic variability among HIV-1 isolates, which is more pronounced in the DNA Pol gene than in the TK gene, and will facilitate considerably the rapid genotypic diagnosis of HSV-1 resistance.

Herpes simplex virus 1 (HSV-1) is a ubiquitous human pathogen throughout the world. After primary infection occurring mostly during infancy, the virus remains latently for life in sensory local ganglia. Thereafter, HSV-1 may be reactivated and can cause usually self-limiting orolabial lesions in immunocompetent individuals. In contrast, immunocompromised patients often develop chronic diseases associated with painful and widespread exanthema and/or enanthema. Since its marketing in the 1980s, acyclovir (ACV), a guanosine analog, has been the drug of choice for the treatment of HSV infections; its active form is ACV triphosphate (1, 2). Foscarnet (FOS), a pyrophosphate analog, directly inhibits the viral DNA polymerase (Pol) and can be used successfully as an alternative drug in case of ACV resistance.

Acyclovir-resistant HSV-1 isolates have a remarkable clinical prevalence, especially in immunocompromised patients. Summarizing published papers of recent years, the prevalence of ACV-resistant HSV strains in immunosuppressed patients ranges between 2.5% and 10.9% (3, 4). For immunocompetent patients, studies between 1985 and 1993 showed that ACV resistance in clinical HSV isolates is $<$1.0%, very low (5–8). An overall increase in ACV-resistant HSV strains could not be established in a subsequent study (9).

The HSV thymidine kinase (TK) protein is responsible for the conversion of ACV to its active form, and 95% of the mutations encoding antiviral resistance are found in the TK gene (10). Only a few cases of resistance are caused by mutations found in DNA Pol, an enzyme catalyzing the elongation of viral DNA (10–12). Mutations can lead to a dysfunctional protein structure, causing loss of enzyme activity or an alteration of substrate specificity. The most frequent resistance-associated mutations are single nucleotide amino acid changes (in the TK or DNA Pol genes) as well as nucleotide deletions (noted by "del" or "Δ") or insertions (noted by "ins" or "::") especially within homopolymer regions, so-called "hot spots," resulting in premature stop codons (the TK gene) (11, 13). Genetic studies revealed that resistance-related mutations are mostly located within active or conserved gene regions (14, 15). Nonetheless, several mutations have been shown to confer resistance despite their location outside conserved gene regions (16). The identification of these mutations is complicated by the high gene variability of TK and DNA Pol (16–19) as well as the complexity and intricacy of drug resistance (2, 20) due to the increasing spectrum of novel mutations in both genes. Thus, a continuous determination of both genotypes is required. Usually, phenotypic HSV-1 assays allow clear interpretation of novel mutations causing drug resistance, but in clinical practice, time-con-

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suming phenotypic testing delays resistance-adjusted treatment. Since there are no equivalent methods for resistance screening that can be used in clinical diagnostics, the comparison of genotype and phenotype is still needed to characterize the resistance association of unknown amino acid changes (21).

The purpose of the present study was to analyze retrospectively the TK and DNA Pol genotypes of 302 clinical HSV-1 strains that were isolated over more than 4 decades from 1973 to 2014, using routine virological diagnostics. All novel, resistant, or unclear genotypes were verified by the corresponding phenotype against ACV and/or FOS. Additionally, the prevalence of natural polymorphisms in the TK and DNA Pol genes was analyzed to check possible changes in clinical HSV-1 isolates over time.

(Data from this study were presented in part at the 25th Annual Meeting of the Society for Virology [GfV], 2015, Bochum, Germany.)

MATERIALS AND METHODS

Patients and viral strains. In this study, 302 clinical HSV-1 cell culture isolates from 289 different patients were included. Strains were collected routinely over 42 years between 1973 and 2014. Samples were taken from 135 female and 139 male patients. In 15 cases, the gender was unknown. The ages of the patients ranged between 13 days and 88 years (mean, 31.1 ± 22.2 years; median, 25 years), whereas information on age was not available from 18 persons. In 159 of 289 (55.0%) patients, the following data about the clinical diagnosis were provided by the clinicians: eczema herpeticum ($n = 49$), herpes genitalis ($n = 25$), herpes facialis ($n = 17$), herpes labialis ($n = 17$), gingivostomatitis ($n = 15$), pneumonia ($n = 9$), meningitis/encephalitis ($n = 8$), keratitis/conjunctivitis ($n = 8$), carditis/myocarditis/pericarditis ($n = 3$), herpes integumentalis ($n = 2$), sepsis ($n = 2$), herpes gestationis ($n = 1$), herpes glutealis ($n = 1$), and pharyngitis ($n = 1$). One patient developed both oral HSV infection and genital herpes. In 130 cases, there was no information about the clinical diagnosis of HSV disease. Twenty-four patients (7.9%) suffered from immunodeficiency. The reasons were acute myeloid leukemia ($n = 5$), acute lymphatic leukemia ($n = 3$), Ewing sarcoma ($n = 1$), and stem cell transplantation (SCT) ($n = 13$). A total of 276 patients were immunocompetent, and for 2 patients, no information about immunocompetence was provided. Clinical resistance to antivirals was diagnosed in seven patients since no improvement of symptoms was observed despite antiviral treatment for at least 10 days. In 6 of them, symptoms did not improve after administration of ACV, and in 1 patient symptoms did not improve during the treatment with FOS. There was no information on any antiviral therapy for the other 282 patients included in this study.

All samples were sent to the Institute of Virology and Antiviral Therapy for virological diagnostics. Herpes simplex virus 1 was identified and HSV-2 could be excluded by diagnostic PCR as described previously (21–23). All HSV-1 strains were isolated and propagated in African green monkey kidney Vero 76 cells (ATCC, CRL 1587) and/or human embryonic lung fibroblasts (HELFs). The method for viral growth has been described previously (19). When the titer reached between 10^6 and 10^8 50% tissue culture infective doses ($TCID_{50}$) ml^{-1} after a maximum of 4 passages, viral stocks were stored at −80°C until use. On average, viral strains were used after one to two passages for geno- and phenotypic testing described below. The exemplary sequence analysis in comparison to a small number of available original samples did not show any differences of TK and DNA Pol sequences.

Genotypic analysis. Viral DNA was prepared from 200 μl of viral stocks with the aid of the QIAamp DNA blood minikit (Qiagen, Hilden, Germany). The TK gene (*UL23*) was amplified in one fragment with a size of 1,131 bp. The DNA Pol gene (*UL30* [3,708 bp]) was divided into four fragments for analysis. Each fragment showed a length of approximately 1,000 bp. The primer sequences were described previously (17, 19) and were based on the sequence of HSV-1 reference strain 17 (GenBank accession no. X14112). For the fourth fragment of the DNA Pol, two novel primers were designed that improved amplification and sequencing results. The two oligonucleotides, with a length of 22 bp each, were Pol-4c (5′-CGA GTG CGA AAA GAC GTT CAC C-3′) and Pol-r1b (5′-G GGT ACC GGT TCG TCG CCC ACG-3′). Both formed a fragment with a length of 1,084 bp.

After DNA amplification, as described earlier (21), amplicons were purified using the QIAquick PCR purification kit (Qiagen). Sequencing was carried out by Eurofins MWG Operons (Ebersberg, Germany). Finally, the alignment of sequence data and the comparison with the published sequence of HSV-1 reference strain 17 (GenBank accession no. X14112) were performed using the software MEGA 5.2. All specified nucleotide sequences corresponded to nucleotide positions in this reference strain.

Phenotypic analysis. Viral strains containing novel, unclear, or resistance-associated mutations in the TK and/or DNA Pol genes were tested phenotypically for their sensitivity toward ACV (GlaxoSmithKline, Uxbridge, United Kingdom). Only isolates with novel or unclear nonsynonymous mutations in the DNA Pol or ACV resistance-associated genotype were tested against FOS (AstraZeneca, Wilmslow, United Kingdom). All phenotypic analyses were performed using a plaque reduction assay, including the formazan test according to the method described previously (16, 17, 19, 21). In short, Vero 76 cells, only used for testing FOS, or human Caucasian fetal lung fibroblasts of the cell line Wi 38 (European Collection of Cell Cultures, Salisbury, United Kingdom), exclusively used for analyzing the phenotype to ACV, were seeded at a density of 10^5 ml^{-1}. After 2 days, the cells were infected with the corresponding viruses at a multiplicity of infection of 0.01. Antiviral agents were added at a final half-log dilution over a range between 0.3 and 35.5 μM ACV or between 13.3 and 844.8 μM FOS. The reference strain HSV-1 MacIntyre (MI [ATCC VR-539]) served as a sensitive control in each experiment. The virus-induced cytopathic effect was assessed microscopically, and a cell proliferation assay using Cell Counting kit-8 (Dojindo Laboratories, Kumamoto, Japan) was carried out. Substance concentrations at half-maximum virus inhibition (50% effective concentration [EC_{50}]) were calculated by linear regression analysis using the software SigmaStat, version 1.01 (Jandel Corporation, San Rafael, CA). For each experiment, resistance to ACV was defined if the mean EC_{50}s of the viral isolates tested were measured at least 5 times higher than the mean value of the included sensitive control strain HSV-1 MI (10). For resistance to FOS, EC_{50}s of ≥330.0 μM were considered (24).

Statistical analysis. Statistical analysis was performed on the basis of 285 strains that were ACV sensitive. Resistant strains were excluded because of their small amount as well as the presence of unknown amino acid substitutions within the TK or DNA Pol. Calculations of polymorphism quantities were carried out using Microsoft Office Excel (Microsoft Corporation). The software SAS 9.4 (SAS Institute, Inc., Cary, NC) and its procedure application GLIMMIX supported the statistical calculations and verifications by means of prevalence regression. The odds ratio (OR) and 95% confidence interval (CI) were calculated to determine the significance of changes within the diversity of substitutions in the time periods 1973 to 1980, 1981 to 1990, 1991 to 2000, 2001 to 2010, and 2011 to 2014. P values of <0.05 were considered significant.

RESULTS

Phenotypic resistance testing. (i) Isolates with an acyclovir- and foscarnet-sensitive phenotype. A total of 103 of 302 (34.1%) HSV-1 isolates were tested phenotypically toward ACV because of novel and/or unclear amino acid changes, frameshifts, or resistance-associated substitutions caused by their TK and DNA Pol genotype. The reference strain HSV-1 MI showed EC_{50}s between 0.1 and 1.8 μM (mean ± standard deviation [SD], 0.6 ± 0.4 μM) for ACV. Thus, the cutoff value for ACV resistance could be calculated as 3.0 μM on average. Eighty-six strains showed ACV susceptibility. The EC_{50}s ranging between <0.1 μM and 2.8 μM

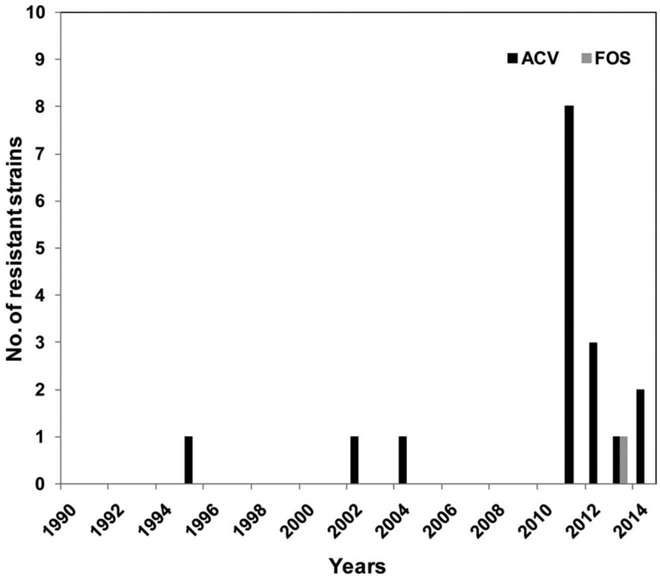

FIG 1 Number of ACV- and FOS-resistant HSV-1 strains over time (1990 to 2014). No resistant strains were detected between 1973 and 1989.

(0.8 ± 0.7 μM) were below the cutoff in each experiment. The susceptibility to FOS was tested for 82 out of 302 (27.2%) HSV-1 isolates, that contained either novel/unclear or resistance-associated mutations in the DNA Pol gene and/or an ACV-resistant phenotype. Out of these, 81 were tested as FOS sensitive, showing EC_{50}s of <330.0 μM.

(ii) Isolates with acyclovir- and/or foscarnet-resistant phenotype. Seventeen of 103 isolates tested were resistant to ACV. The EC_{50}s of ACV ranged between 12.9 μM and ≥35.5 μM, in comparison to 0.3 to 1.7 μM (0.8 ± 0.6 μM) for the sensitive reference strain HSV-1 MI. In addition, one ACV-resistant isolate (no. 176-13, ACV EC_{50} of 3.2 μM versus 0.4 μM for HSV-1 MI) was tested as resistant to FOS (mean EC_{50} of 422.4 μM versus 121.8 μM for HSV-1 MI). In a summary of these results, 5.6% (17/302) of all HSV-1 strains of this study were ACV resistant. In the immunocompetent patients, 4.3% (12/276) of all strains were resistant to ACV. In contrast, 25% (6/24) of HSV-1 isolates obtained from immunocompromised patients conferred ACV resistance, and 6 of 13 (46.2%) patients who underwent SCT had ACV-resistant HSV-1 strains. The numbers of ACV- and FOS-resistant HSV-1 strains over time are shown in Fig. 1.

Genotypic resistance testing. (i) Thymidine kinase. In TK, 26 novel amino acid substitutions related to the natural gene polymorphism as well as two hitherto unknown deletions (Δ35Q and Δ36K) without resistance association were analyzed. Additionally, the known (16) but so far unclear substitution A118V could be characterized as a natural polymorphism (Table 1). Furthermore, the amino acid change A156V, which was reported previously as being associated with ACV resistance (16), was detected in one strain (no. 185-93) with an ACV-sensitive phenotype. In addition, nine already published resistance-associated substitutions and frameshifts were related to the TK gene (Table 2). Furthermore, one novel amino acid change (E257K) and one unknown frameshift (CAGCTTTCGGGGGΔ; nucleotides [nt] 781 to 793 resulting in a stop at amino acid position 263) leading to ACV resistance were identified. The resistance associations of five novel amino acid substitutions (Q15K, E43A, P269S, S276N, and I326V) and of the known but hitherto unclear substitution A93V (25) remained unclear, whereby Q15K, E43A, A93V, and I326V emerged in parallel in a single ACV-resistant strain (no. 01-14). Moreover, E43A, P269S, and S276N were encoded in the TK gene of three further ACV-resistant strains (no. 459-11, 474-11, and 242-12) in addition to known resistance-associated mutations. All novel nonsynonymous mutations in the TK gene were located outside conserved or active gene regions (Tables 1 and 2 and Fig. 2A).

(ii) DNA polymerase. In DNA Pol, 69 so far unknown amino acid substitutions and two deletions (Δ667E and Δ668G) without a premature stop codon were identified as result of the natural gene polymorphism. Three of them were related to the conserved regions IV (G458S), ΔC region (K591R), and VII (Y946F). Six known but so far unclear amino acid changes (L267M [26], M356I [18], E662D [21], E860K [27], T1121M [21], and M1226I [21]) were characterized as associated with ACV susceptibility (Table 3 and Fig. 2B). Surprisingly, the strain (no. 976-04) with the substitution S775N, which has been published as conferring resistance (18), was tested as ACV sensitive (Table 3). Additionally, the known amino acid change S724N leading to both ACV and FOS resistance was detected in one HSV-1 strain (no. 176-13) (Table 4). G901V was characterized as novel resistance-associated substitution (no. 288-95). The significance of eight novel amino acid changes (E70K, G191S, L359I, L448V, G691S, A748T, G841A, and P1114H) remained unclear since resistance-associated nonsynonymous mutations were present in the TK or DNA Pol genes (Tables 2 and 4). Two of them (G191S and P1114H) were detected in the same strain (no. 459-11) together with novel substitutions

TABLE 1 Phenotype associated with novel or known but hitherto unclear amino acid substitutions/frameshifts within the TK of 103 clinical HSV-1 isolates

Novel substitution(s)/frameshift(s)[a]	Mean ± SD EC_{50} of ACV (μM)[b]	Phenotype to ACV	Resistance association
H7P, A12T, R18C, R20H, R26H, Δ35Q, Δ36K, L72V, R75C, A98T, <u>A118V</u>,[c] A137T, V138F, A156P, A156T, <u>A156V</u>,[c] D215A, A233S, P268M, G271D, N277S, N277K, A309V, A316V, G335D, G346W, T350I, V352I, D363N, G373E	<0.1 to 2.8 ± 0.3	Sensitive	No
E257K, S263stop (del nt 781–793)	≥35.5	Resistant	Yes
Q15K, E43A, <u>A93V</u>,[d] P269S, S276N, I326V	12.9 ± 0.4 to ≥35.5	Resistant	Unclear[e]

[a] Hitherto unclear amino acid substitutions/frameshifts are underlined.
[b] Two independent experiments were performed to derive the mean.
[c] Published substitution from reference 16.
[d] Published substitution from reference 25.
[e] The resistance association of these substitutions could not be clarified.

TABLE 2 TK genotype of 17 ACV-resistant HSV-1 strains

Strain no.	Polymorphism(s)[a] Natural	Associated with resistance	Unclear significance	Phenotype (mean ± SD EC_{50} of ACV, μM)[b]
288-95	N23S, K36E, L42P, A265T			15.0 ± 4.8
496-02	N23S, R32C, K36E, R89Q	*C del nt 548–553*		13.3 ± 0.7
1686-04	C6G, N23S, K36E, L42P, A265T	Y172C		≥35.5
76-11	C6G, N23S, K36E	G ins nt 430–437 D228stop		≥35.5
116-11		G del nt 430–436 M182stop		20.9 ± 4.0
152-11	N23S, K36E, R89Q	G ins nt 430–437 D228stop		≥35.5
217-11	N23S, K36E, A265T	C ins nt 548–554 D228stop		≥35.5
258-11	N23S, K36E, R89Q	C ins nt 548–554 D228stop		≥35.5
401-11	N23S, K36E, R89Q, G240E, A265T	**E257K**		≥35.5
459-11	C6G, N23S, K36E, L42P	A del nt 184–187 M85stop	**E43A**	≥35.5
474-11	S276R	R216H	**P269S**	12.9 ± 0.4
59-12	R20S, N23S, K36E, R89Q	*C del nt 548–553*		≥35.5
242-12	N23S, K36E, R89Q, G251C, A265T	R222H	**S276N**	≥35.5
264-12	N23S, K36E, A265T	T287M		≥35.5
176-13[c]	N23S, K36E, I78F, R89Q, G240E, S332L			3.2 ± 0.2
01-14	C6G, N23S, K36E, L42P, A265T		**Q15K, E43A, A93V, I326V**	≥35.5
18-14	N23S, K36E, I78F	**CAGCTTTCGGGGG del nt 781–793 S263stop**		≥35.5

[a] Novel amino acid substitutions are in bold, and deletions (del) without a premature stop codon are in italic. ins, insertion.
[b] The mean ± SD EC_{50} of ACV for the sensitive control strain HSV-1 MI is 0.6 ± 0.2 μM. The cutoff value for ACV resistance is 3.0 μM. Two independent experiments were performed to derive the mean.
[c] This strain has additional FOS resistance. The mean ± SD EC_{50} of ACV for the sensitive control strain HSV-1 MI is 0.4 ± 0.1 μM. The cutoff value for ACV resistance is 2.0 μM. Two independent experiments were performed to derive the mean.

(TK, E43A, unclear; DNA Pol, A870V, natural polymorphism) in addition to a deletion of one adenine in the TK (A del nt 184 to 187 leading to M85stop [Tables 2 and 4]). With the exception of two substitutions (L448V encoded in the conserved region IV and G841A encoded in the conserved region III), all nonsynonymous mutations clustered outside conserved DNA Pol gene regions (Fig. 2B).

Distribution of amino acid changes over time. Table 5 shows the prevalence of natural polymorphisms in the TK and DNA Pol genes over time from 1973 to 2014. The average number of TK polymorphisms ranged from 5.38 (1973 to 1980) to 6.45 (2011 to 2014), and that of the polymorphisms in the DNA Pol differed between 4.95 (1973 to 1980) and 5.50 (1981 to 1990) per strain. Between 0.38 (2001 to 2010) and 0.91 (2011 to 2014) different amino acid substitutions per strain were observed in TK and between 0.61 (2001 to 2010) and 0.86 (2011 to 2014) in DNA Pol. Statistical analysis revealed that there was no significant difference ($P = 0.91$; coefficient within 95% CI, −0.01 [−0.026 to 0.024]; OR, 1) in the number of natural TK and DNA Pol polymorphisms from 1973 to 2014. Overall, the four most common nonsynonymous natural polymorphisms in 285 ACV-sensitive HSV-1 isolates of this study were A265T (99.3%), N23S and K36E (both 95.1%), and R89Q (55.4%) in TK, as well as A330R (100%), S33G (91.6%), V905M (84.2%), and T1208A (78.9%) in DNA Pol. Compared to the periods prior to 2011 to 2014, G251C replaced R89Q as one of the most frequent substitutions of TK.

DISCUSSION

The findings of this study confirm the high genetic variability of TK and DNA Pol of HSV-1. Although this topic has been ad-

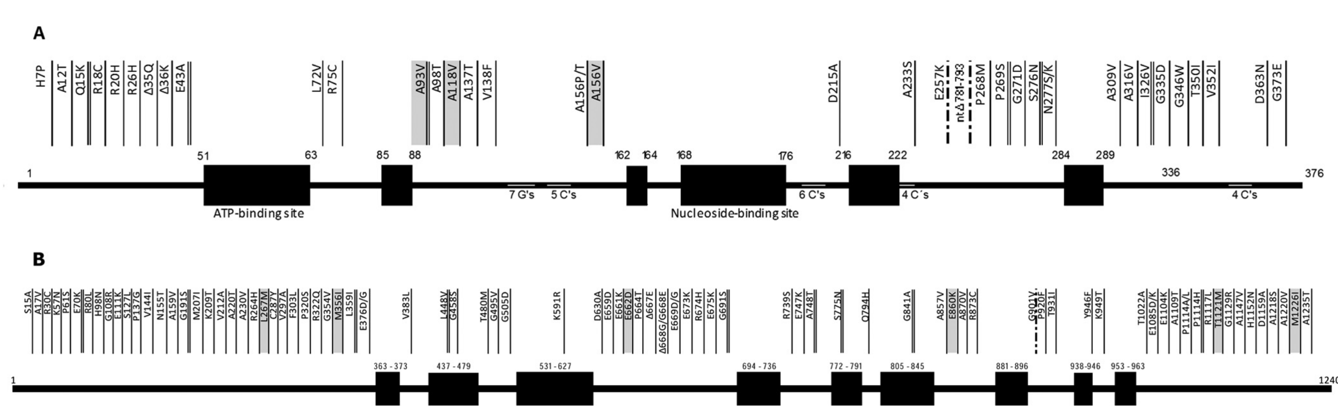

FIG 2 HSV-1 TK (A) and DNA Pol (B) genes with novel natural polymorphisms marked as continuous lines, novel resistance-associated mutations marked as broken lines (dots and dashes), and unclear amino acid substitutions marked as double lines, along with novel nucleotide deletions or insertions found in this study. Substitutions already published are shaded in gray.

TABLE 3 Phenotype associated with novel or known but hitherto unclear amino acid substitutions within the DNA Pol of 103 phenotypically tested clinical HSV-1 isolates

Novel substitution(s)[a]	Mean ± SD EC_{50} of ACV (μM)[b]	Phenotype to ACV	Resistance association
S15A, A17V, R30C, K57N, P61S, R80L, H98N, G108R, E111K, S127L, P137G, V144I, N155T, A159V, M207I, K209T, V212A, A220T, A230V, R264H, L267M,[c] C287Y, V297A, F303L, P320S, R322Q, G354V, M356I,[d] E376D, E376G, V383L, G458S,* T480M, G495V, G505D, K591R,* D630A, E659D, E661K, E662D,[e] P664T, Δ667E, Δ668G, G668E, E669D, E669G, E673K, R674H, E675K, R739S, E747K, Q794H, A857V, E860K,[f] A870V, R873C, P920F, T931I, Y946F,* K949T, T1022A, E1085D, E1085K, E1104K, A1109T, P1114A, P1114L, R1117L, T1121M,[e] G1129R, A1147V, H1152N, D1159A, A1218S, A1220V, M1226I,[e] A1235T	<0.1 to 2.8 ± 0.3	Sensitive	No
S775N*[d]	2.8 ± 0.3	Sensitive	Unclear[g]
G901V	15.0 ± 4.8	Resistant	Yes
E70K, G191S, L359I, L448V,* G691S, A748T, G841A,* P1114H	12.9 ± 0.4 to ≥35.5	Resistant	Unclear[g]

[a] Hitherto unclear amino acid substitutions are underlined. *, substitutions in conserved regions.
[b] Two independent experiments were performed to derive the mean.
[c] Known substitution from reference 26.
[d] Known substitution from reference 18.
[e] Known substitution from reference 21.
[f] Known substitution from reference 27.
[g] The resistance association of these substitutions could not be clarified.

dressed in many investigations, this study reports on a considerable number of novel nonsynonymous mutations after screening of more than 300 HSV-1 strains isolated routinely between 1973 and 2014. Twenty-six novel amino acid substitutions and two unknown deletions without resistance association could be analyzed in the TK protein alone. None out of them was encoded in conserved gene regions. This was also the case for two novel resistance-associated substitutions. Thus, the present results underline the necessity to characterize nonsynonymous mutations, especially outside conserved or active regions for their relationships to drug resistance. A noteworthy example is an exchange of alanine (Ala) at TK position 156 showing a high diversity. Altogether, the present results show that Ala can be replaced in clinical HSV-1 strains by proline (Pro) or threonine (Thr), resulting in no alteration of TK function. However, in a recent study, the substitution of Ala by valine (Val) was characterized as most likely conferring ACV resistance (16). After TK phosphorylation activity had been shown for this HSV-1 isolate (28), the present study demonstrates clearly that the substitution A156V, comparable to A156P and A156T, is connected with an ACV-sensitive HSV-1 phenotype (no. 185-93) and therefore is not related to any resistance.

For DNA Pol, 69 novel amino acid substitutions related to

TABLE 4 DNA polymerase genotype of 17 ACV-resistant HSV-1 strains

Strain no.	Polymorphism(s)[a]: Natural	Associated with resistance	Unclear significance	Phenotype (mean ± SD EC_{50} of FOS, μM)[b]
288-95	S33G, A330R, V905M, E1104D, P1124H, T1208A	**G901V**		105.6 ± 7.4
496-02	S33G, A330R, D672N, V905M, A1203T			82.5 ± 8.7
1686-04	S33G, V905M			30.7 ± 3.1
76-11	S33G, A330R, V905M, S1123L, P1124H, T1208A		**G691S, G841A**	65.7 ± 26.2
116-11	S33G, A330R, V905M, S1123L, P1124H, T1208A			227.7 ± 9.6
152-11	S33G, E70K, A330R, V905M, P920S, P1199Q, T1208A			94.4 ± 7.0
217-11	S33G, A330R, V905M, A1203T, T1208A			NT[c]
258-11	S33G, A330R, V905M, A1203T, T1208A			124.4 ± 31.4
401-11	A330R, P1124H, T1208A			212.2 ± 32.3
459-11	S33G, A330R, A566T, **A870V**, V905M, P1124H, T1208A		**G191S, P1114H**	106.3 ± 12.3
474-11	S33G, C287Y, A330R, V905M, D1103H, P1124H, T1208A		**A748T**	106.3 ± 10.7
59-12	S33G, A330R, **E661K,** G749D, P1199Q, T1208A			81.5 ± 12.6
242-12	S33G, A330R, V905M, P1124H, T1208A			134.7 ± 0.8
264-12	S33G, A330R, V905M, P1124H, T1208A		**E70K, L359I**	264.7 ± 7.5
176-13[d]	S33G, A330R, V905M	S724N		**≥330.0**
01-14	S33G, A330R, V715A, A870V, V905M			175.9 ± 16.0
18-14	S33G, A330R, V905M, A1203T, T1208A		**L448V**	143.2 ± 4.2

[a] Novel amino acid substitutions are in bold.
[b] The mean ± SD EC_{50} of FOS for the sensitive control strain HSV-1 MI is 172.8 ± 52.8 μM. The cutoff value for FOS resistance is ≥330.0 μM. Two independent experiments were performed to derive the mean.
[c] NT, not tested.
[d] This strain has additional FOS resistance.

TABLE 5 Distribution of amino acid substitutions recognized as natural polymorphisms encoded in the TK *(UL23)* and DNA Pol *(UL30)* genes of 285 ACV-sensitive HSV-1 isolates over the time period from 1973 to 2014

Time period	Absolute diversity (diversity/strain)		No. of natural polymorphisms/ strain		Four most frequent aa substitutions (no. of HSV-1 isolates)		Total no. of HSV-1 isolates
	UL23	*UL30*	*UL23*	*UL30*	*UL23*	*UL30*	
1973–1980	20 (0.95)	15 (0.71)	5.38	4.95	A265T (21), N23S (20), K36E (20), R89Q (12)	A330R (21), S33G (20), V905M (19), T1208A (17)	21
1981–1990	23 (0.44)	38 (0.73)	5.81	5.50	A265T (52), N23S (46), K36E (46), R89Q (24)	A330R (52), S33G (46), V905M (46), T1208A (43)	52
1991–2000	41 (0.42)	61 (0.63)	6.16	5.39	A265T (97), N23S (92), K36E (92), R89Q (52)	A330R (97), S33G (90), T1208A (75), V905M (73)	97
2001–2010	35 (0.38)	57 (0.61)	5.69	5.28	N23S (92), K36E (92), A265T (91), R89Q (63)	A330R (93), S33G (83), V905M (83), T1208A (72)	93
2011–2014	20 (0.91)	19 (0.86)	6.45	5.36	A265T (22), N23S (21), K36E (21), G251C (9)	S33G (22), A330R (22), V905M (19), T1208A (18)	22

natural gene polymorphism and two so far unknown deletions without a premature stop codon could be analyzed. However, three of the novel substitutions were encoded in the conserved regions Exo II (G458S), Exo III (K591R), and VII (Y946F), and the viral replication was not affected. These findings are partly in contrast to the findings from Gibbs et al. (29), who have described that amino acid substitutions related to the Exo II region strongly affect DNA Pol activity, and the virus fails to replicate. Conversely, Gilbert et al. (30) indicated that resistance-associated mutations are usually not located within the Exo II region. Whereas all three substitutions found to be encoded in conserved regions of DNA Pol gene had an ACV-sensitive phenotype, the novel amino acid change G901V encoded outside conserved regions conferred ACV resistance. Considering the available published data and the results of this study, no reliable correlation can be made between the localization of amino acid substitutions and their influence on ACV susceptibility of the corresponding HSV-1 strain without the comparison of genotype and phenotype (16, 30). Surprisingly, the known resistance-related substitution S775N (18) caused by a mutation in the conserved DNA Pol region VI was classified in the present study as a natural polymorphism. Due to this discrepancy, the significance of this amino acid change has to be verified by recombinant phenotyping using the bacterial artificial chromosome (BAC) technology (31). Furthermore, a probable association of resistance to ACV of the novel amino acid change G841A, which was detected in addition to the unknown substitution G691S (DNA Pol) and the previously published insertion of guanine at nt 430 to 437 (TK) in an ACV-resistant but FOS-sensitive isolate (76-11 [Tables 2 and 4]) could not be clarified. However, the similar substitutions G841S (32) and G841C (33) have been proved to confer ACV (G841S, G841C) and FOS (G841C) resistance.

The analysis of the numerical distribution of natural polymorphisms within the TK and DNA Pol genes revealed that there were no differences over time from 1973 to 2014. Nevertheless, small changes in the type of amino acid substitutions could be detected. In TK, the substitutions N23S, K36E, A265T, and R89Q are the most frequent changes since 1973 and the substitution G251C replaced R89Q between 2011 and 2014. For DNA Pol, A330R, S33G, V905M, and T1208A have to be considered the most frequent substitutions. In previous publications, a similar distribution of the four frequent TK (19) or DNA Pol (16, 17) polymorphisms could be analyzed. Whereas the absolute numbers of polymorphisms in the TK (5.5 to 7.0) and DNA Pol (5.0 to 5.5) genes per strain are comparable over 40 years, DNA Pol shows in principle a greater genetic variability because of the larger amount of different intragenic polymorphisms.

It is remarkable that ACV-resistant strains (in total, 17/302 [5.6%]) occurred only from 1995 onwards in this study retrospectively analyzing routinely collected HSV-1 isolates from 1973 to 2014. The highest number of 8 ACV-resistant HSV-1 strains could be detected in 2011, and in 2013, the only strain (no. 176-13) conferring resistance to both ACV and FOS was obtained. These findings suggest that the number of patients developing ACV resistance has increased during the last 2 decades, but resistance against FOS is quite rarely observed. Unfortunately, no certain statement can be made about the development of resistance over the time due to the low number of ACV-resistant strains (17/302 [5.6%]) in this study. Nevertheless, the findings confirm that ACV resistance appears predominantly in immunosuppressed patients with SCT. Hence, from 6 of 13 (46.2%) patients who underwent SCT, ACV-resistant HSV-1 isolates were obtained. In 2014, Frobert et al. (27) reported an increase in the prevalence of ACV resistance in allogeneic hematopoietic SCT patients from 14.3% between 2002 and 2006 to 46.5% between 2007 and 2011. In contrast, 12 ACV-resistant HSV-1 strains (4.3%) were isolated from 276 immunocompetent patients of this study. In such patients, a low prevalence of ACV resistance between 0.3 and 0.7% has been described (4, 9, 34, 35). Conversely, Wang et al. (36) reported an unexpectedly high rate of 4.0% ACV-resistant HSV-1 strains in immunocompetent children with oral herpetic lesions, and Duan et al. (37) described a prevalence rate of 6.4% ACV-resistant HSV-1 strains in patients with recurrent herpetic keratitis. However, it has to be considered for the interpretation of the present findings that there are some limitations regarding the scarce information about clinical diagnosis, immunocompetence of patients, antiviral therapy modalities, and longitudinal observations. The primary goal was to present a genotyping summary of TK and DNA Pol in 302 HSV-1 isolates collected over more than 4 decades in routine HSV diagnostics. Furthermore, the virus stocks used for geno- and phenotypic resistance testing were not plaque purified since this method is not established in routine virological diagnostics. It is known that clinical specimens may contain a mixture of wild-type virus and minor variants conferring drug resistance, which can be lost during cultivation in cell culture and/or cannot be detected by basic sequencing methods.

In summary and conclusion, by retrospective analysis of 302 HSV-1 isolates collected over more than 4 decades, this study characterized a high number of novel natural amino acid polymorphisms and several novel resistance-associated substitutions in TK and DNA Pol. The findings confirm the strong genetic variability, which is more pronounced in DNA Pol than in TK, and will facilitate considerably the rapid genotypic diagnosis of HSV-1 resistance. However, the definitive assignment of novel resistance mutations requires recombinant phenotyping. Nevertheless ACV-resistant strains occur predominantly in the immunocompromised, in particular in patients undergoing SCT, whereby the number of patients with resistant HSV-1 strains has increased during the last 2 decades.

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